

**COMPARISON OF SALIVARY AND SERUM LEVELS OF SOLUBLE TOLL-
LIKE RECEPTOR 3 (TLR3) IN PATIENTS WITH SJÖGREN'S SYNDROME
(SS) COMPARED TO AGE- AND SEX-MATCHED CONTROLS**

A Thesis

by

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ABSTRACT

This cross-sectional study examined potential differences in concentration of Toll-like receptor 3 (TLR3) in serum and citric acid-stimulated saliva from patients with Sjögren's Syndrome (SS) and healthy age-, sex-, and periodontal disease- matched control patients. Salivation was stimulated with 5 ml of 2% citric acid, and whole saliva samples were collected. Full-mouth clinical periodontal measurements were recorded from 21 SS patients and 22 unaffected control patients. Saliva and plasma TLR3 concentrations were determined by enzyme-linked immunosorbentassays (ELISA). Data were tested statistically as appropriate to the data distribution.

No significant difference in clinical periodontal measurements, or serum or salivary TLR3 concentrations was evident between groups. Salivary flow rates were significantly lower ($p<0.001$) whereas plaque % were significantly higher ($p=0.048$) in SS patients. SS patients expressed on average 0.183 and 0.219 ng/ml TLR in saliva and serum, respectively, while healthy control patients expressed on average 0.299 and 0.188 ng/ml TLR in saliva and serum, respectively. These differences were not significantly different ($p<0.05$). This is the first detection of TLR3 in human serum or saliva as quantified or qualified by ELISA.

The present findings do not support a hypothesis that a hyperinflammatory systemic state, as seen in SS patients, can independently raise serum or saliva TLR3 concentration in the absence of periodontal disease in treated SS patients.

DEDICATION

To God, the Father, the Son, and the Holy Ghost.

Praise God, from whom all blessings flow;

Praise Him, all creatures here below;

Praise Him above, ye heavenly host;

Praise Father, Son, and Holy Ghost. Amen.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I.1 SJÖGREN'S SYNDROME OVERVIEW

Sjögren's Syndrome (SS) is a chronic, systemic autoimmune disorder. The course of the disease destroys salivary and lacrimal glands, and manifests diverse findings and symptoms, ranging from exocrinopathy to extraglandular, as well as pulmonary, hepatic, renal, and neurologic systemic involvement. Genetic, environmental, and hormonal factors may trigger SS. The precise etiology, or etiologies, has yet to be determined. B-lymphocyte hyper-reactivity, autoantibody production and T-cell lymphocytic infiltration to exocrine glands, and commonly, other organs, characterize the pathosis. The hallmark symptoms of SS are decreased exocrine gland function resulting in xerostomia and xerophthalmia, referred to collectively as 'Sicca Syndrome' ¹.

In the United States, 2-4 million people are estimated to have SS, while only 1 million have an established diagnosis. SS may go undiagnosed due to the varied and confounding range of clinical manifestations. SS has a predilection for women, with a female:male ratio of 9:1. Its onset is most commonly in the 4th to 6th decades of life. SS may be classified as either primary SS (pSS) or secondary SS (sSS). sSS is more common, and is defined by SS with a concomitant autoimmune disorder such as systemic sclerosis, rheumatoid arthritis, or systemic lupus erythematosus. Perhaps 25% of rheumatoid arthritis and systemic lupus erythematosus patients present histologic

evidence of SS ². Approximately 60% of SS is secondary. pSS exists independently in the absence of other autoimmune disorders ³. Globally, epidemiologic data gathered on SS has shown significant variability. This variability may result from methodological inconsistencies between studies. Criteria for disease classification in studies differ, as do the methods used for quantitative testing of lacrimal and salivary gland function ^{4,5,6}. Even within the same country, independent investigations have reported prevalence with 10-fold differences ⁷. Collectively, the prevalence of pSS appears highest in northern Europe, lowest in Asia, and approximately equal in North America and continental Europe ⁸. Prevalence ranging from 0.1-4.0% has been reported, with annual incidence from 3.9/100,000-5.3/100,000 ⁸⁻¹³.

I.2 CLINICAL SYMPTOMS

The hallmark symptoms of SS, xerostomia and xerophthalmia, may result in additional acute and chronic manifestations. Xerostomia may precede polydipsia, dental complications, oral candidiasis, angular cheilitis, and parotiditis. Xerophthalmia may precede corneal injury and recurrent ocular infection ¹⁴.

Respiratory, gastrointestinal and integumentary exocrine involvement is less common. However, this involvement may result in symptoms of nasal crusts and bleeds, parotid and submandibular swelling, dry throat, cough, esophageal mucosal atrophy, atrophic gastritis and dyspareunia. Extraglandular symptoms are not uncommon, and have been observed in up to 55% of SS patients ¹⁵. Systemic manifestations are more common in younger SS patients ¹⁶.

SS patients report symptoms of chronic fatigue, low-grade fever and myalgias, arthralgias, and other organ involvement. Significant fatigue, which is often the chief complaint, occurs in half of patients with pSS ¹.

Cutaneous manifestations of SS include xerosis, angular cheilitis, eyelid dermatitis, pruritus, cutaneous vasculitis (often as palpable purpura), and erythema annulare ¹⁷. Cutaneous rashes of SS often cannot be distinguished clinically or histologically from subcutaneous lupus erythematosus lesions ¹⁸. Bernacchi et al., quantified cutaneous manifestations of SS, reporting that xerosis is the most frequent and characteristic cutaneous manifestation of pSS (56.4%) and sSS (25.8%) ¹⁹. Flat purpura and hypergammaglobulinemia are often concomitant, while palpable purpura is associated with dermal vasculitis. Dermal vasculitis is linked with SS-related extraglandular manifestations and lymphoma ²⁰. In a recent study of pSS, 89 of 559 patients (16%) presented with cutaneous involvement. Cutaneous vasculitis was the most common cutaneous involvement (58%). Small vessel, leukocytoclastic vasculitis was predominant. Vasculitis is associated with extraglandular and immunologic SS features. Clinically, small vessel vasculitis presents as palpable purpura, urticaria or erythema multiforme ²⁰.

Arthritis independent of or present with arthralgia are frequently diagnosed as well. Following a 4.5-year observation of 102 patients with pSS, 73.5% reported arthralgia and 17.6% demonstrated arthritis. The ankles, metacarpal and metatarsal joints, shoulders and wrists were the most commonly affected joints ²¹.

Muscle pain is common in pSS. In a study of 48 pSS patients, 44% complained of muscle pain, while 27% fulfilled the American College of Rheumatology criteria for fibromyalgia. Of the 36 muscle biopsies obtained for the study, 26 (72%) showed signs of inflammation. Abnormal expression of major histocompatibility complex (MHC) class I, MHC class II and MAC was found in 50%, 44%, and 75% of pSS biopsies, thus histopathologic signs of myositis are common ²².

Neurological manifestations of SS are not uncommon, and are well documented. SS patients most commonly have a sensory neuropathy affecting the feet and lower legs. This neuropathy affects light touch, proprioception and vibratory sensation. Manifestations of peripheral neuropathy include sensory ataxic neuropathy, painful sensory neuropathy without sensory ataxia, multiple mononeuropathies, multiple cranial neuropathy, trigeminal neuropathy, autonomic neuropathy, and radiculoneuropathy ²³.

In a study of pSS patients conducted by Scofield et al., of 12 pSS positive for both anti-Ro and anti-La, 8 (66.7%) had neuropathy. Among 76 of 88 pSS patients studied with anti-Ro or anti-La positivity, 19 (25%) had neuropathy. Overall, approximately 30% of pSS patients displayed a pure sensory neuropathy ²⁴. A study conducted by Gono et al., observed 47% of the pSS patients participating displayed pure sensory neuropathy ²⁵. However, the incidence of these observations contrasts drastically with those from Pavalakis et al., who observed approximately 1% displaying sensory neuropathy ²⁶.

Although pSS incidence in patients with interstitial lung disease (ILD) is not significantly elevated, patients with pSS who have ILD likely have increased premature

mortality ²⁷. Hypergammaglobulinemia and lymphopenia, positivity for RF, anti-La and anti-Ro, and impaired forced vital capacity and/or forced expiratory volume in 1 second values may be predictive parameters with a high specificity for detection of pSS-associated lung disease ²⁸.

Renal pathosis is a documented finding in SS ²⁹. Chronic tubulointerstitial nephritis is the predominant kidney biopsy finding in patients with renal involvement from pSS ³⁰. An interstitial lymphocytic infiltrate with tubular atrophy and fibrosis is the most commonly reported histopathologic lesion. This lymphocytic infiltrate correlates with tubular dysfunction ²⁹. Pertovaara reported that high levels of serum total gamma-globulin, serum protein and serum beta2m were the best predictors of the development of distal renal tubule acidosis in pSS patients ³¹.

Common gastrointestinal symptoms include dysphagia, dysmotility and gastritis. Dyspepsia was reported in 23% of pSS patients ³². A Hungarian study found the frequency of celiac disease to be approximately 10 times higher in pSS patients than non-SS patients ³³.

Jara et al., described thyroid dysfunction in SS patients, reporting that pSS was ten times more frequent in patients with autoimmune thyroid disease, and autoimmune thyroiditis was nine times more frequent in pSS. Those who suffer from pSS were most likely to develop hypothyroidism among any who have common autoimmune diseases ³⁴.

Approximately 5% of SS develop lymphoproliferative disorders. B-cell hyperactivity explains increased prevalence of these diseases ³⁵. The odds ratio of

lymphoma was 4.75 in pSS and 9.57 in sSS ³⁶. Palpable purpura, low C4 levels, and mixed monoclonal cryoglobulinaemia are risk factors for the comorbidity of lymphoma and SS ^{1, 37}.

I.3 DIAGNOSIS OF SJÖGREN'S SYNDROME

Diagnosis of SS is multifaceted and difficult. To date, no one sign, symptom, or lab value is exclusive to SS. Standardization of diagnostic criteria of the disease has only recently developed. The two prevailing diagnostic criteria are the European classification criteria and the European classification criteria as revised by the American European Consensus (AECC) group ^{38, 39}.

The hallmark symptoms of xerostomia and xerophthalmia must be present in addition to laboratory findings. The revised AECC criteria are reportedly more specific, however less sensitive than the European classification criteria in the diagnosis of pSS. The specificity and sensitivity of the European classification criteria are 75% and 65.7%, respectively, compared to 97.2% and 48.6%, of the AECC. When applied to sSS, specificity of the two classification systems is equal, and the European classification criteria remain more sensitive (70.6% versus 64.7%) ⁴⁰.

If SS is suspected, other symptomatic etiologies causing xerostomia and xerophthalmia must be considered in a differential diagnosis. Appropriate exclusion criteria including use of anticholinergic drugs, hepatitis C and HIV infection, lymphoma, sarcoidosis, graft-versus host disease and previous head and/or neck radiation. Further complicating diagnosis, 'sicca syndrome' symptoms may be mimicked by amyloidosis, trauma, and scarring. Chronic blepharitis or conjunctivitis, neurogenic

impairment of eyelid function or lacrimal gland dysfunction may result in xerophthalmia. Diabetes mellitus, hypercalcaemia and multiple medications may cause xerostomia ¹⁴.

Thus, an unequivocal diagnosis of SS requires subjective patient report as well as multiple objective parameters. Non-organ specific antigens including immunoglobulins, alpha-fodrin ⁴¹, nuclear antigens, and extractable nuclear and cytoplasmic antigens are targeted by autoantibodies ¹⁴. Borg et al., reported anti-Ro/SS-A and/or anti-La/SS-B in 80%, immunoglobulin M-Rheumatoid factor (IgM-RF) in 68%, and anti-nuclear antibody (ANA) in 77% of 65 SS studied ⁴². Anti-Ro/SS-A and anti-La/SS-B are included in the diagnostic criteria for SS. Anti-Ro/SS-A is more commonly found in SS, however, anti-La/SS-B is considered more specific. Once a definitive diagnosis of SS is made, anti-Ro/SS-A and anti-La/SS-B may be used to monitor disease activity and progression. These antibodies correlate with disease severity and activity. Anti-Ro/SS-A and anti-La/SS-B may serve as predictors for future SS in the absence of symptoms as well ⁴³. However, absence of anti-Ro/SS-A and/or anti-La/SS-B does not preclude a diagnosis of SS.

I.4 IMMUNE DYSREGULATION IN SJÖGREN'S SYNDROME

Both innate and adaptive immune system alteration has been documented in SS. Though the factor initiating salivary gland destruction remains to be discovered, it is speculated that viral infection serves as an impetus. Viral infection may be a font of antigenic ligands to Toll-like receptors (TLRs), located on dendritic and epithelial cells of the salivary glands. Viral ligands binding to TLRs initiate an immune cascade. TLRs

are activated, presenting MHC class 2 molecules and secreting cytokines and chemokines including Type-1 IFN ¹⁴.

The involvement of Type-1 IFN in the development and progression of SS has been thoroughly investigated. Microarray data from minor salivary glands, peripheral blood mononuclear cells, and whole and peripheral blood CD14+ monocytes revealed that Type-1 IFN inducible pro-inflammatory and anti-viral genes were among the mostly up-regulated genes in SS patients. Over-expression of Type-1 IFN-inducible genes also correlated with the presence of antibodies to Ro/SSA and La/SSB antigens. Notably, IFN-induced genes include B-cell activating factor (BAFF) ⁴⁴. Plasmacytoid dendritic cells, which have been identified in the salivary glands of SS patients, produce Type 1-IFN. IFN-inducible genes IFITM1 and BAFF were highly expressed in those glands as well ⁴⁵.

BAFF levels are significantly increased in SS. This over-expression is implicated in B-cell tolerance breakdown in SS. Aberrant B-cell distribution, hyperactivity and autoantibody production are abnormalities associated with over-expression. Monocytes and epithelial cells produce BAFF. BAFF stimulates B-cell survival ⁴⁶. Seropositivity for anti-Ro/SS-A or anti-La/SS-B has been correlated with increased BAFF levels ⁴⁷. Increased BAFF has also been correlated to known lymphoproliferative complications in SS including B-cell lymphoma. Other cytokines reputed to be involved in SS pathogenesis include interleukin(IL)-6, IL-12, IL-17 and IL-21. An in vivo animal model has demonstrated IL-12 induced salivary gland damage ¹⁴.

Secretion of cytokines following innate immune system activation precipitates migration of T and B lymphocytes from serum to the salivary gland parenchyma. Lymphocyte invasion starts at the periductal area and spreads as pan-lobular invasion. CD8 T-cells are present, though CD4 T-cells predominate. Antibody-secreting B-cells comprise a minority of infiltrating cells ⁴⁸. T-cell activation in SS has been highly associated with increased IL-6. Increased IL-6 may increase Th-17, which belongs to a pro-inflammatory subset of CD4 T cells, in salivary glands. Th17 cells secrete IL-17, a pro-inflammatory cytokine implicated in multiple autoimmune disorders ⁴⁹. The invading lymphocytes are then activated by epithelial and dendritic cells, which act as antigen presenting cells within the glands. Ro/SS-A and La/SS-B, alpha and beta fodrin and cholinergic muscarinic receptor antigens constitute the specific antigens presented in SS ¹⁴.

An alternative mechanism of salivary gland pathogenesis is a proposed immune-mediated neurotransmission alteration. Dawson, et al., demonstrated in vitro that immunoglobulin G (IgG) from pSS patients contains putative anti-muscarinic antibodies directed against muscarinic M₃ receptors. Normally, binding of acetylcholine to M₃ parasympathetic receptors would result in salivary production. However, the anti-muscarinic IgG antibodies inhibited production ⁵⁰. Damage to muscarinic receptors by autoantibodies may precipitate and precede exocrinopathy. Parasympathetic nerves innervate the salivary glands. Therefore, damage to M₃ muscarinic receptor by autoantibodies would alter acetylcholine binding from parasympathetic nerves. Indeed, antibodies to M₃ have been documented in SS patients ⁴¹.

The spatial location of water channel proteins, including aquaporins (AQP), on epithelial cells may affect salivary secretion as well. Normally located on the apical membrane of acinar cells, an AQP5 isoform was located on the basolateral membrane in pSS patients. In these patients, treatment with anti-tumor necrosis factor (TNF)-alpha antibody repositioned AQP5 to its normal apical position ^{51, 52}. This positional restoration was accompanied by improvement of salivary flow.

Anti-Ro/SS-A and anti-La/SS-B have been and continue to be an essential component of SS. Ro/SS-A antigens include Ro52 and Ro60. These antigens were once considered as a uniform, coupled system. Chan, et al., demonstrated that they are distinct, encoded by different genes ⁵³. Ro60 is a 60 kDa protein component of a small cytoplasmic ribonucleoprotein complex ⁵⁴. Ro52 is a 52 kDa RING/Bbox/coiled-coil (RBCC) tripartite motif protein (TRIM), and an over-expressed ubiquitin-ligase in peripheral blood mononuclear cells in SS and systemic lupus erythematosus patients ^{55, 56}. The anti-Ro/SSA proteins negatively regulate cytokine production induced by the IFN-gamma pathway by functioning as an E3 ligase ⁵⁷. La antigen is a 47 kDa protein involved in RNA polymerase III transcription, tRNA biogenesis, mRNA stabilization, and translational regulation ⁵⁸. Accurate detection of these antibodies remains difficult. If Ro/SS-A samples contain both Ro52 and Ro60 and a classical SS-A ELISA is used for quantification, up to 20% of Ro52 or Ro60 positive samples may remain undetected ⁵⁷.

Research by Ramos-Casals et al., noted that patients with a young onset of pSS, defined as younger than 35 years old, presented a higher prevalence of anti-Ro/SS-A antibodies than an patients with onset after 35 (70% vs 28%) ⁵⁹. When compared to

younger pSS patients, pSS patients with disease onset occurring after 70 years old, the prevalence of anti-Ro/SS-A or anti-La/SS-B was similar ⁶⁰. When pSS is compared by gender, some studies report men may have a lower anti-Ro/SS-A and/or anti-La/SS-B antibody frequency ^{61, 62}. However, this is not a unanimous observation ⁶³.

The literature is more united regarding antibody presence and extraglandular clinical course. Patients possessing these antibodies display parotiditis and extraglandular complications more frequently than those who do not ⁶⁴⁻⁶⁷. Loch et al., concluded that anti-La/SS-B are strong predictors of internal organ affection in pSS, whereas anti-Ro/SS-A are not associated with complications of the kidneys, lungs or liver among pSS patients ⁶⁶.

The relationship between antibodies and lymphoma is not yet understood. Davidson et al., reported that 3 of 100 pSS patients studied developed non-Hodgkin lymphoma during a 10 year observation period ⁶⁸. All 3 were anti-Ro/SS-A and anti-La/SS-B positive. Conversely, an Italian study found that of pSS patients with non-Hodgkin's lymphoma, 37% were anti-Ro/SS-A and 50% anti-La/SS-B positive ⁶⁷.

Toker et al., documented anti-Ro/SS-A and anti-La/SS-B autoantibodies in the lacrimal tears of pSS, and a correlation of these autoantibodies with rose Bengal staining scores, Schirmer I test score, and xerophthalmia symptoms ⁶⁹. Markuse et al., echoed Toker's report of a correlation, and also reported a correlation between anti-Ro/SS-A and an abnormal sialogram ⁷⁰. Tsuzaka et al., observed severe parotid swelling and salivary gland dysfunction among pSS patients with serological abnormalities with anti-Ro/SSA and with or without anti-La/SS-B ⁷¹. Among pSS patients, the degree of

infiltration in the labial salivary gland tissue was significantly greater in patients with anti-Ro/SS-A plus anti-La/SS-B antibodies in their sera than in patients with anti-Ro/SS-A antibodies alone ($p < 0.05$)⁷².

Serological abnormalities including hypergammaglobulinemia^{73, 74}, hypocomplementemia^{75, 76}, high rheumatoid factor, high serum levels of IgG and IgA⁷⁷,⁷⁸, mixed monoclonal IgM cryoglobulins⁷⁹, and positive ANA⁸⁰ titers are well established associations with pSS and seropositivity for anti-Ro/SS-A and anti-La/SS-B.

1.5 PERIODONTAL DISEASE AND SJÖGRENS SYNDROME

The relationship between SS and periodontal disease is the subject of investigation and debate. Clinical, microbiological, and biochemical studies have been conducted to elucidate whether or not a relationship exists, and, should it exist, what its nature is. A viral etiology may be shared by both periodontal disease and SS. Evidence of this will be addressed following the presentation of literature which investigated the simultaneous incidence, prevalence, and disease progression of periodontal disease and SS.

Clinical studies comparing SS patients to healthy controls have reported varied findings. Antoniazzi et al., observed that pSS and sSS patients, as diagnosed by the European Community criteria for SS, had significantly higher mean plaque index (PI), gingival index (GI), probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) than SS-free controls. Only GI remained significantly higher in patients with SS once adjustment for plaque was made. Subjects with pSS showed lower gingival crevicular fluid (GCF) IL-1beta levels compared to SS-free controls⁸¹. Najera

et al., qualified SS using the same criteria as Antoniazzi and compared the periodontal status of SS patients to SS-free controls. SS patients had significantly higher PI, decayed/missing/filled teeth, increased alveolar bone loss, CAL and cement-enamel junction to-bone crest loss. Najera did not find any significant difference in the number of cases of “established periodontitis” between SS and SS-free subjects. However, he concluded that odds ratio analysis suggested SS patients are at 2.2 higher risk of periodontal disease than SS-free ⁸². Though which criteria used for SS diagnosis was not disclosed, Celenligil et al., recorded clinical periodontal parameters as well as serum IgG antibody levels of a panel of 13 oral microorganisms in pSS and sSS patients, as well as healthy controls. Significantly higher PI, BOP, PD, and PI scores were observed in SS patients. SS patients also exhibited significantly elevated antibody levels to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. The authors thus purposed that SS may affect bacterial colonization in plaque and contribute to increased periodontal disease ⁸³.

Conversely, Kuru et al., reported that the occurrence, severity and extent of periodontal lesions were not significantly different between pSS, sSS and SS-free patients. PI, GI, BOP, PD, CAL, as well as other parameters were essentially equivalent. Furthermore, microbiological assay of subgingival dental plaque samples by Periocheck® and polymerase chain reaction (PCR) analysis showed no difference in frequency of periodontal pathogens. The authors posited that these results neither supported a SS-specific clinical and microbiological periodontal status requiring special periodontal care, nor that the periodontal status of patients with SS was significantly

different from healthy SS-free control ⁸⁴. Schiødt et al., concluded that pSS is not associated with increased risk of periodontal disease. Fifty-seven pSS patients and 80 SS-free age-matched controls were evaluated clinically. pSS was qualified using the Copenhagen criteria. The Danish study found no significant difference in any clinical periodontal parameter ⁸⁵. Using the American-European Consensus criteria for pSS, 20 pSS patients were compared to 20 age-matched healthy controls. As expected, salivary flow rates were lower among pSS patients. Excluding a slightly higher GI, the periodontal parameters measure did not differ significantly between groups. Furthermore, PI, GI, and PD were not correlated to salivary flow rates ⁸⁶. An earlier study by Pedersen qualifying pSS using the European consensus criteria found similar results ⁸⁷. Boutsis et al., found no difference in the clinical periodontal parameters between pSS/sSS patients, SS-free patients with other immune diseases and SS-free/immune disease free patients with subjective xerostomia ⁸⁸. When comparing the periodontal parameters of pSS patient to SS-free/xerostomic and SS-free/periodontal disease patients, Pers et al., reported that despite a significantly lower PI in pSS patients, the three groups did not differ with respect to modified GI, papillary bleeding, or PD. The PI of pSS patients did not correlate with other periodontal variables. Compared to SS-free groups, pSS patients did exhibit a significantly higher salivary level of BAFF ⁸⁹. Jorkjend et al., found that SS patients stratified by salivary output, as compared to SS-free xerostomic patients stratified by salivary output, showed no statistically significant differences in periodontal status as measured by attachment loss, leading the authors to suggest that SS patients do not have an increased risk for developing periodontitis ⁹⁰.

A small clinical study by Tervahartiala et al., assessed the periodontal status and collagenase, elastase and gelatinase in gingival crevicular fluid in patients with SS, periodontal disease, and healthy SS-free/periodontal disease-free controls. Of note, SS was diagnosed using the Copenhagen criteria. The collagenase and elastase activity was significantly lower in SS patients and controls than SS-free patients with periodontitis. Gelatinase profiles were similar among all groups. Though a visible plaque index, gingival bleeding index, and pocket depth were reportedly assessed for each patient, the results were not reported. The author commented that, “With one exception, the clinical status in SS was comparable to that found in control subjects with healthy periodontium and oral mucosa, and unlike that you in adult patients with periodontitis”⁹¹. Ravald et al., investigated dental caries, periodontal parameters, stimulated and unstimulated saliva in patients with pSS and healthy controls. All periodontal parameters measured, including PD, furcation involvement, bleeding on probing and PI did not differ significantly, which Ravald notes is in agreement with Tervahartiala’s findings as well as Tseng’s findings⁹². Tseng determined the periodontal status of 14 SS and 14 SS-free age-matched female. PI, GI, bleeding on probing, calculus, CAL and PD were recorded on 7 test teeth. There were no statistically significant differences between the SS and control groups. Tseng concluded that Sjögren's syndrome has no observable influence upon the indices measured in evaluating periodontal disease⁹³. In a study investigating periodontal status and sulcular *Candida albicans* colonization in pSS patients, pSS patients had significantly great CAL as compared to SS-free controls. PD, GI and PI were not significantly different. The increased attachment loss in pSS patients was not

attributed to an increase in colonization of the gingival sulci by *Candida albicans* organisms⁹⁴.

Alteration of vascular anatomy in patients with SS is documented by studies utilizing nail fold capillaroscopy⁹⁵. Whether the digital alterations are pathognomonic of SS or present in a subset of patients is debated⁹⁶. Other authors explain that patients with SS have a normal morphologic pattern of capillaries, but that there is an abnormal macrovascular and microvascular reactivity^{97, 98, 99}. The morphology of oral vasculature has been investigated as well. Scardina et al., compared the capillaroscopic microcirculation pattern of the interdental papilla of patients with SS and healthy controls. The capillaries of SS patients exhibited significantly greater apical, afferent and efferent capillary diameter, capillary density and capillary tortuosity, creating a pathognomonic periodontal SS vascular morphology. Only the length of the visible capillary loops was not significantly different between SS and controls. This forms a cobweb conformation rather than a more typical punctuated pattern¹⁰⁰. A similarly distinct pattern of microvascular alteration was described when capillaroscopy was applied to the mucosa of the lower lip corresponding to the frenulum¹⁰¹.

I.6 SJÖGREN'S SYNDROME AND VIRAL INFECTION

A viral pathogenesis of SS has been proposed. Salivary gland immune response in SS is characterized by IFN signature. This signature includes over-expression of IFN-inducible genes including interferon-stimulated transcription factor-3-gamma (ISF3G) and IFN-induced transmembrane proteins (IFITM1). Hjelmervik et al., used a 16K complementary DNA microarray to generate gene expression profiles in minor salivary

glands (MSGs) obtained from 10 patients with primary SS (pSS) and 10 control subjects. Quantitative reverse transcriptase-PCR validated the results. The authors found a distinct difference in gene expression levels of MSGs. Among the differences between pSS and controls was the increased expression of numerous type-1 IFN genes related to virus infection as compared to controls ¹⁰². Work by Gottenberg et al., supported Hjelmervik's findings ¹⁰³.

The cause of cellular activation and the chronic inflammatory response that targets epithelial cells in SS is not known. There is evidence that a persistent viral infection may cause pSS. Work by Fox et al., drew attention to Epstein-Barr virus (EBV) as a possible initiation factor in pSS pathogenesis. Salivary gland biopsies were performed on 14 pSS patients, and salivary samples taken from 20 pSS patients. In 8 of 14 biopsies, cytoplasmic staining of ductal and/or acinar epithelial cells with monoclonal antibody against the EBV-encoded early "D" antigen was noted. This antibody was non-reactive with any other tissues obtained from pSS. The absence of late structural antigens like gp200/350 negate a lytic EBV infection. Eight of 20 salivary samples from pSS contained detectable EBV. EBV was not detectable in any pSS-free participants. Furthermore, presence of EBV was associated with clinically more severe SS. The authors hypothesized that EBV-associated antigens in the salivary gland may serve as a target for immune recognition and lymphoproliferation by T- and/or B- cells, causing infiltration and destruction of salivary glands ¹⁰⁴. Saito et al., extracted DNA from peripheral blood mononuclear cells (PBMC) and salivary gland biopsy tissue from SS and SS-free controls. Following PCR, the frequency of SS salivary glands containing

EBV DNA was 78%. 18% of SS PBMC contained EBV DNA. SS-free controls expressed 13% frequency in salivary glands and 6% in PBMC ¹⁰⁵. In situ hybridization and PCR was used by Mariette et al., to detect EBV DNA in salivary gland biopsy specimens from pSS and sSS patients as well as SS-free controls. In situ hybridization revealed EBV DNA in the epithelial cells of biopsy specimens in 50% of pSS, none in sSS, and 8% of SS-free controls. 86% of pSS, 60% of sSS and 29% of SS-free patients were EBV DNA positive as detected by PCR. A statistically significant increase in EBV DNA of pSS as compared to SS-free controls was revealed by both detection techniques ¹⁰⁶. The association of EBV with SS was further supported by the results of Wen et al., who detected EBV genomes and their products in epithelial cells and lymphocytes in over half of the salivary glands of SS patients studied. EBV genome was not present in any SS-free control specimens ¹⁰⁷.

The detection of EBV DNA in primarily or exclusively SS salivary glands has not been observed by all studies. Maitland et al., examined labial minor salivary gland biopsies for EBV and human cytomegalovirus (HCMV) DNA by PCR in patients with sSS and SS-free non-specific sialadenitis patients. No significant difference between the detection of EBV or HCMV in salivary gland biopsies from sSS patients or SS-free patients. Of note, the SS patients in the study conducted by Fox were not specified as pSS or sSS. Furthermore, the controls were disease-free and without sialadenitis ¹⁰⁸. Venables et al., examined 12 minor and labial salivary gland biopsies from 12 pSS and 10 SS-free controls for EBV DNA. Utilizing in-situ hybridization and

immunofluorescence, DNA was identified in 2 of 12 pSS patients and 6 of 10 controls. Of the 6 control biopsies, 5 were without inflammation or Class II expression ¹⁰⁹.

While EBV has been identified in SS in some studies, its existence in either a lytic or latent state is debated. Late structural protein gp320/330 was not expressed in some studies ^{110, 107}. Positive staining in epithelial cells and scattered B-lymphocytes for the protein product of BamHI fragment Z, ZEBRA, were observed in other studies. ZEBRA is believed to convert EBV from a latent to a lytic state ^{109, 110}.

When the evidence of EBV as a causative agent in SS is weighed, it is, as a body, inconclusive. The unique role of EBV in SS, if any, remains to be clearly defined. Other viruses have been considered as pathogenic agents as well.

Human T lymphotropic virus type 1 (HTLV-1), human immunodeficiency virus (HIV), intracisternal A type particles, and human retrovirus 5 (HRV-5) have been associated with pSS. An infectious acquired genome, HRV-5, was discovered by reverse transcriptase PCR from the salivary gland tissue of a SS patient. The 932-bp retrovirus sequence is related to type B and D retroviruses ¹¹¹. Following the discovery of HRV-5 in a SS-positive biopsy, Rigby et al., examined 93 minor salivary gland tissue samples and found no association of the virus with SS ¹¹². HRV-5 was later quantified in synovial sample for arthritic joints, blood samples from rheumatoid arthritis and systemic lupus erythematosus ¹¹³. Even if HRV-5 is not itself directly or uniquely involved in the pathogenesis of SS, retroviruses may cause proliferation of endogenous and defective retroviral RNAs ¹¹⁴.

A-type retroviruses were described as those with a characteristic intracellular morphology of a thick shell with an electron-lucent, hollow center. These characteristic structures are now recognized to be an intermediate, immature viral capsid transitioning to more mature structures. 'A-type retrovirus' is still used to describe endogenous non-infectious retrotransposon structures now referred to as intracisternal type A particles (IAPs). IAPs conduct the assembly of morphologically-immature virus-like particles. These particles are not released efficiently extracellularly, budding into the lumen of the endoplasmic reticular cisterns ¹¹⁵. Garry et al., observed IAPs in T-lymphoblastoid cell cultures treated with tissue homogenates from pSS salivary glands. pSS homogenate treated cultured cells were then examined by transmission electron microscopy and determined to express viral particles similar to HIV. These viral particles could then possibly serve as an antigenic stimulus for production of glycosaminoglycan (Gag)-reactive antibodies in sera from patients suffering from autoimmune disorders ¹¹⁶. Using an immunoblotting assay, PCR, reverse transcriptase activity assay, and transmission electron microscopy, Yamano et al., also observed unknown IAPs in labial salivary glands and peripheral blood mononuclear cells of pSS ¹¹⁷. Sanders reported that patients with autoimmune disorders display antibodies against IAP proteins ¹¹⁸.

Single stranded RNA viruses have been implicated in SS as well. Triantafyllopoulou et al., provided evidence that pSS may be associated with coxsackievirus infection of minor salivary gland epithelial cells and local lymphocytic infiltrates. Coxsackieviruses are single-stranded, non-enveloped family picornaviridae RNA viruses. The authors applied a differential display protocol and semi-nested reverse

transcriptase-PCR to MSG RNA samples from pSS, sSS, SS-free/autoimmune disease-positive, and SS-free/healthy controls. 11 of 12 pSS, 1 of 13 sSS, and no controls were positive for the presence of coxsackievirus genome or the main antigenic capsid protein VP1. The amplification products were identical to the coxsackieviral serotypes B4 and A13 ¹¹⁹. However, Triantafyllopoulou's et al., findings could not be duplicated by Gottenberg et al., ¹⁰³.

Triantafyllopoulou et al., posited the continued study of coxsackieviruses as possible pathogens in pSS. He argued that significant evidence exists that coxsackieviruses establish persistent non-cytolytic infections in vitro, resulting in autoimmune induction as has been observed in type 1 diabetes and autoimmune dilated cardiomyopathy ¹²⁰. Next, Triantafyllopoulou argued that host-cell protein interaction with coxsackievirus may be related to autoimmunity induction. Translocation of the La protein from the nucleus to the cytoplasm has been shown following enterovirus cleavage of the La protein ¹²¹. This translocation is considered essential for the recognition of La as an autoantigen. Finally, B-cell polyclonal activation may be triggered by persistent noncytopathic RNA viral infection of epithelial cells, as has been demonstrated by HCMV infection of pancreatic islet cells and the observed polyclonal hyper-gammaglobulinemia and autoantibody production ¹²².

I.7 PERIODONTAL DISEASE AND VIRAL INFECTION

The proposed causes of periodontal disease are many. The body of literature on periodontal pathology has focused primarily on bacterial etiology. However, investigation of viruses in periodontal disease suggests concomitant viral infection.

EBV, HCMV, and HSV as well as other common viruses have been the focus of several studies as possible etiologic agents. These studies have collected virus-positive samples from subgingival plaque samples, gingival crevicular fluid, tissue biopsies, as well as serum and saliva samples. International, independent scientific investigation continues to add evidence of a viral pathogenesis.

Contreras et al., investigated the frequency of HCMV, EBV-1, EBV-2, herpes simplex virus (HSV) and human immunodeficiency virus (HIV) by means of a nested-PCR. Subgingival specimens were obtained by paper-point sampling from deep (7-9 mm) sites and shallow (3-5 mm) sites. Of 27 participants, HCMV was observed in 14, EBV-1 in 10, HSV in 7, EBV-2 in 6 and HIV in 1. 89% of participants were positive for at least one virus in deep sites. By comparison, 56% of shallow sites were positive. Also, viral co-infection was four times as common in deep sites ¹²³. When classified as periodontally healthy, chronic periodontitis, and aggressive periodontitis, Das et al., found that HSV-1 and EBV are significantly associated with chronic and aggressive periodontitis, and that when present in aggressive periodontitis, HSV-1 was associated with sites with increased probing depth and CAL ¹²⁴. Among the Japanese population studied by Kato et al., EBV DNA was found more frequently in patients with PD at least 5 mm deep (deep sites) rather than PD no more than 3 mm (shallow sites). Furthermore, EBV was present with *P. gingivalis* in 40% of deep sites versus 14% of shallow sites. Furthermore, the odds ratio of EBV DNA in patients with deeper sites was 2.36 of EBV DNA. This was 2.07-fold greater than those with shallow sites. Collectively, Kato et al., posits that EBV DNA may be a pathogenic factor in chronic periodontitis ¹²⁵.

Viral pathogens have been detected in the periodontal anatomy as well. Madinier et al., used a Southern blot with ³²P-radiolabeled DNA probes to screen intra- and extra-oral tissue samples for EBV DNA. EBV DNA was present in the interproximal papilla samples obtained. Only 1 of 20 extra-periodontal mucosal samples contained homologous EBV DNA. These periodontal status of the teeth from which the surrounding tissue samples were obtained was not recorded ¹²⁶. Vincent-Bugnas et al., reported, for the first time in the literature, EBV-infected junctional and sulcular epithelial cells in periodontally diseased tissue. EBV was detected not only in periodontally diseased sites but healthy sites as well. The level of EBV infection positively correlated with disease severity. Vincent-Bugnas observed that EBV-infected epithelial cells are typically in a latent state, as evidenced by the presence of latent transcripts including EBNA1, EBNA3, LMP1, and LMP2. It was also observed that infection of sulcular and junctional epithelium cells with EBV was associated with apoptotic cell death ¹²⁷. The observation of viral pathogens in these structures may be important to better understanding periodontal regeneration.

Few studies have investigated how the presence of viral pathogens affects the outcome of periodontal therapy. Bertoldi et al., investigated whether the presence of herpes simplex 1 and 2 (HSV-1 and HSV-2) affected the clinical outcomes of surgical periodontal regeneration in isolated deep intraosseous defects free of common periodontopathogenic bacteria. Patients diagnosed with generalized chronic periodontitis with no less than one deep intraosseous defect were selected. Following non-surgical therapy, and whole mouth surgical therapy everywhere but the deepest intraosseous

defect, a base line examination and microbiological profile of each participant was completed. Only patients free of periodontogenic bacteria, including *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Treponema denticola*, *Gusobacterium nucleatum*, and *Prevotella intermedia* were included. The minimally invasive surgical technique (MIST) with application of Emdogain® was then applied to the remaining intraosseous defect. One year following surgical therapy, HSV-1 and HSV-2 were quantified in the pocket tissues associated to the intraosseous defects. Of the 17 defects treated, 5 were positive for HSV-1. HSV-2 was not detected in any site. CAL gain and PD reduction were significantly worse in patients who tested positive for HSV-1 following surgical intervention as compared to HSV-1 negative patients. Gingival recession was greater among HSV-1 positive patients as well. As the number of HSV-1 DNA copies increased, the amount of CAL gain and PD reduction decreased, gingival recession increased as well. HSV-1 negative patients gained 5.92 +/- 1.93 mm CA, while HSV-1 positive patients gained only 0.2 +/- 1.79 mm ¹²⁸.

Some studies have explored viral DNA in salivary samples. Idesawa et al., observed that the stimulated whole saliva of 48.5% of patients with periodontitis was EBV positive. Comparatively, the saliva of 15% of healthy subjects was positive. Periodontitis was defined as at least two clinical probing depths greater than 4 mm. Significantly less BOP was observed among EBV negative periodontitis patients as compared to EBV positive periodontitis patients. Following initial, non-surgical periodontal therapy, a significant reduction in EBV positive patients, EBV levels, and

BOP was observed ¹²⁹. Bilder et al., examined unstimulated saliva samples from chronic periodontitis patients, with at least 2 teeth with at least 6 mm CAL and at least 5 mm PD. Two periodontally healthy groups were included as well, distinguished by whether or not they belonged to a dental profession. Fifteen percent, 5%, and 40% of salivary samples from chronic periodontitis patients tested positive for HCMV, HSV-1, and EBV, respectively. Neither healthy group was HCMV positive. Otherwise, virus frequency was not significantly different among groups ¹³⁰. Dawson et al., tested the hypothesis that salivary levels of EBV and HCMV DNA correlate with periodontal disease severity. Additional criteria to qualify patients as periodontally diseased included PD no less than 5 mm, at least 3 mm CAL, and BOP. Of note, patients with salivary gland dysfunction were excluded. Following collection of 5 ml of unstimulated saliva, periodontal examinations were performed. All participants were diagnosed with moderate or severe periodontal disease. EBV and HCMV DNA were detected in the saliva of 81.5% and 1.5% of patients, respectively, and in the subgingival plaque samples of 44.6% and 0.3% of patients, respectively. The presence of EBV in saliva did not correlate with increasing severity of periodontal disease, though it did increase the chance of the presence of subgingival plaque ten-fold ¹³¹. Sahin et al., investigated EBV and HCMV as well. In addition to participants with periodontal disease, Sahin included participants with gingivitis and edentulous patients. Among periodontitis participants, 79% were EBV positive. Gingivitis and edentulous patients were 33% and 54% EBV positive, respectively. One-half of patients with periodontitis were HCMV positive. Conversely, gingivitis and edentulous patients were EBV free. Dentate patients were at an increased

risk for HCMV (OR=1.318), but not EBV. From these results, Sahin posited that his results suggested periodontitis lesions were the main source of salivary HCMV, as all other patient variables were controlled and the groups were equally matched ¹³². Sugano et al., collected stimulated whole saliva from subjects with periodontitis, defined as at least two sites with PD greater than 4. The saliva was then analyzed by PCR for *P. gingivalis* and EBV. 48.5% of patients were EBV positive, and *P. gingivalis* was significantly higher than in EBV negative salivary samples. No clinical measurements or data reflecting disease severity were reported ¹³³.

Not all studies have reported high subgingival virus prevalence. Upon analysis of subgingival plaque samples, no HCMV was detected in chronic periodontitis, localized or generalized aggressive periodontitis, or healthy controls by Nibali et al., EBV was detected in 25% of localized aggressive periodontitis patients, but was undetected in chronic periodontitis patients. Furthermore, EBV detection was not statistically significantly different between any periodontitis group and healthy controls ¹³⁴.

Bilichodmath et al., reported the prevalence of herpesviruses in chronic and aggressive periodontitis patients in an Indian population. Subgingival plaque specimens were collected and processed by PCR. 26.32%, 100%, 15.7% and 78.9% of chronic periodontitis patients were HCMV, HSV-1, HSV-2 and EBV positive, respectively. Aggressive periodontitis patients were 7.14%, 57.14%, 0%, and 28.57% positive for HCMV, HSV-1, HSV-2 and EBV, respectively. The presence of HCMV, HSV-1, and EBV was statistically significant in chronic periodontitis patients, but was not significant among aggressive periodontitis patients. The presence of HSV-2 was not statistically

significant in either chronic or aggressive periodontitis patients ¹³⁵. Imbronito et al., completed a similar study in Brazil. Because the disease definitions and viral detection methods were similar, comparison between the study results is possible. In addition to investigation of the prevalence of herpesviruses in chronic and aggressive periodontitis patients, herpesvirus prevalence in gingivitis and disease-free patients was also investigated. The patients were diagnosed using the same American Academy of Periodontology diagnostic criteria as applied in the Bilichodmath et al., study. Following nested PCR, Imbronito et al., reported that 50%, 40% and 46% of chronic periodontitis patients were HCMV, HSV-1, or EBV positive. Aggressive periodontitis patients were 46.7%, 86.7% and 33.3% positive for HCMV, HSV-1, and EBV, respectively ¹³⁶. The sample size per disease category was greater in the Imbronito study, with 30 participants per category, compared to less than 20 chronic or aggressive periodontitis patients in the Bilichodmath study. Imbronito reported HCMV, HSV-1, and EBV in 56.7%, 20%, and 0% of healthy patients, respectively, and 40%, 53%, and 20%, respectively, of gingivitis patients. As compared to chronic periodontitis and disease-free patients, the frequency of HSV-1 in aggressive periodontitis patients was significantly greater. The frequency of HCMV was not different between groups, while EBV-1 frequency was not significantly different between periodontitis/gingivitis groups and disease-free controls ¹³⁷. Yapar et al., investigated HCMV and EBV in aggressive patients and reported 64.7% were HCMV positive, while 70.6% were EBV-1 positive. No disease-free controls were HCMV positive, and only 6.3% were EBV positive. All aggressive patients were treated with surgical therapy. However, viral frequency was evaluated only prior to therapy and

only clinical parameters were reported as indication of response to therapy ¹³⁰. Grenier et al., demonstrated that viruses may be eliminated by non-surgical therapy, and evaluated GCF of healthy, gingivitis, and periodontitis patients by nested PCR amplification. HCMV prevalence was significantly higher in periodontitis as compared to periodontal health. Furthermore, as probing depth increased, HCMV incidence increased among periodontitis patients. Finally, of 48 study participants, 8 agreed to undergo non-surgical scaling periodontal therapy. Sites were evaluated for the presence of HCMV, EBV and HSV prior to and 6 week following therapy. 7, 1 and 4 of these patients were HCMV, EBV and HSV positive prior to treatment, respectively. At the time of re-evaluation, a single patient was HSV positive only ¹³⁸.

I.8 TOLL LIKE RECEPTOR 3

Toll-like receptors (TLRs) are pathogen recognition receptors that recognize multiple distinct pathogens associated molecular patterns (PAMPs). TLRs were first discovered in flies and later in humans. To date, 11 distinct TLRs have been discovered in humans. It has been established that TLRs play a critical role in the initiation of an innate immune response. Research suggests that they may play a role in the adaptive immune response as well. TLRs are type-1 membrane glycoproteins with PAMP-recognizing extracellular leucine-rich repeats and cytoplasmic Toll/IL1 (TIR) receptor domain. The known TLRs are divided into 5 subfamilies based on amino acid sequence and genomic structure. TLR3 is unique among TLRs, with five exons, four of which encode its protein. All human TLR proteins except TLR3 proteins are encoded by no more than two exons. TLRs 3, 7, 8, 9 recognize viral and bacterial nucleic acid, while

TLRs 1, 2, 4, 5, 6, 10 recognize PAMPs associated with bacteria, fungi and protozoa. Upon recognition of PAMPs, TLRs play a critical role in the initiation of an immune response ¹³⁹.

TLR3 recognizes the PAMP viral double-stranded RNA (dsRNA). dsRNA is produced during viral replication as either part of viral RNA genome or part of the replication cycle. Viral dsRNA, as well as endogenous dsRNA released from apoptotic cells activates TLR3. Polyriboinosinic:polyribocytidylic acid (polyI:C) is a synthetic dsRNA analogue and TLR3 ligand often used to study TLR3. Immune cells including dendritic cells, macrophages, natural killer cells, and mast cells express TLR3. Initially thought to be expressed exclusively intracellularly, mounting evidence indicates it is expressed extracellularly as well ¹⁴⁰⁻¹⁴².

The macro structure of TLR3 is divided into three anatomic and functional sub-structures defined by their spatial relationship to the membrane TLR3 traverses. The ectodomain contains multiple leucine-rich repeats (LRRs) and is responsible for ligand binding.

Among these LRRs, LRR 12 and LRR 20 are unique to TLR3. A total of 23 LRRs are arranged into a polarized, heavily glycosylated solenoid (coil). This solenoid structure is bent into a horseshoe configuration with specialized domains capping the N- and C- terminal ends of the solenoid. The outer, convex surface of the horseshoe structure is positively charged. The inner, concave surface is negatively charged and hydrophobic. The hydrophobicity results from the inward orientation of the consensus leucine side chains of the LRRs. An extended parallel beta-sheet forms the concave

surface. The beta-sheet is comprised of beta-strands contributed from each of the eight residues of each LRR motif. A single disulfide bond stabilizes the hairpin look of the N-terminal motif. The C-terminal contains an internal alpha helix stabilized by 2 disulfide bonds. The horseshoe conformation is planar rather than twisted, as would be anticipated by constituent beta-sheet twist. The planarity is thought to result from irregularities in the loops, which connect beta strands of LRRs 8, 12, 14, 18, and 20. Short alpha helices and a large conserved loop at LRR 20 protrude from convex faces of the ectodomain. A large conserved loop formed by beta strands protrudes from LRR 12. The planarity of the ectodomain facilitates ligand binding and receptor signaling. The ectodomain is highly glycosylated, with carbohydrates apportioned on the convex, concave, and one of the two lateral surfaces. The opposite, non-glycosylated surface presents a large area for molecular interaction. A total of three points of contact are necessary between each TLR3 structure and dsRNA molecule for binding: the N-terminal, C-terminal, and C-terminal dimerization site. An inverse relationship between TLR3/ligand binding in an environmental pH greater than 6 exists. Beyond pH 6.5, TLR3 will not bind its ligand. dsRNA must be at least 45 base pairs long to bind to its receptor and trigger an activation signal ¹⁴³⁻¹⁴⁵.

TLR3 binds dsRNA in dimeric pairs at three sites of interaction. Two sites bind dsRNA while one forms the dimer ¹⁴⁶. As the length of the ligand increases, so does the number of dimers bound to the ligand. As Liu described, the TLR3 ectodomain does not undergo a conformational change once bound to dsRNA. Two TLR3 ectodomains at the C-terminal ends to form a dimer. The glycan-free lateral surface of each horseshoe-

shaped ectodomain interacts only with the ribose-phosphate backbone of the dsRNA ligand. Thus, TLR3 binds dsRNA independent of ligand base sequence ¹⁴⁷.

Once the bound to its ligand and dimerized, the Toll-II-1 receptor (TIR) is reoriented. This reorientation recruits the adapter molecule TIR domain-containing adapter inducing IFN- β (TRIF). TRIF then connects to protein kinases TAK1, IKK and TBK/IKK ϵ . The subsequent activation of transcription factors IRF3, NF-kappaB, and AP-1 results in induction of IFN genes as well as hundreds of other cellular genes ^{148, 149}.

Yamashita observed TLR3 mediated signaling is not always preceded by gene induction, and signaling affects cellular properties independently of TRIF and MyD88 adaptor proteins. The proto-oncoprotein Src is auto-phosphorylated by the TLR3-dsRNA complex. Initially, this complex causes cell migration, proliferation and adhesion. However, lipid rafts contained in the plasma membrane sequester Src and inactivate it, causing inhibition of cell migration, proliferation and adhesion ¹⁵⁰.

Alexopolou first observed that mammalian TLR3 recognizes dsRNA, and that the activation of the receptor induces NF-kB and the production of type I interferons (IFNs). In the novel study, polyinosine-polycytidylic acid (poly(I:C)) was used as a synthetic ligand for TLR3 ¹⁵¹. dsRNA-induced up-regulation of co-stimulatory molecules was not entirely dependent on TLR2-Trif axis. dsRNA induced up-regulation of costimulatory molecules were entirely dependent on type I interferon receptor signaling. They found that genetic mutation in murine genes encoding Trif and TLR3 had a strong inhibitory effect on dsRNA -induced synthesis of TNF and type I

interferon ¹⁵². Matsukura showed that TLR3 was expressed inside airway epithelial cells and up-regulates expression of inflammatory cytokines and chemokines through the TLR3-NF- κ B and TLR3-IRF3 signaling when stimulated by poly(I:C) ¹⁵³. Matsumoto et al., observed poly(I:C) induction of NF- κ B and IFN- β . The recognition of dsRNA by TLR3 was highly specific, discriminating between the presence and absence of a hydroxyl group bound to the 2' carbon in beta-d-ribose ¹⁵⁴. Duffy et al., created a monoclonal antibody (mAb) that recognizes the extracellular domain of human TLR3 in a conformation-dependent manner. Application of this mAb to poly(I:C) stimulated human lung epithelial cells results in down-regulation of IL-6, IL-8, MCP-1, RANTES, and IP-10 production, as well as NF- κ B, IRF-3/ISRE, and p38 MAPK ¹⁵⁵.

A mAb recognizing the extracellular domain of murine TLR3 was developed by Bunting et al., and applied to a murine model *in vivo*. This anti-TLR3 antibody resulted in concentration-dependent inhibition of poly(I:C)-induced NF- κ B activation and chemokine generation. This finding further validated previously described TLR3-elicited inflammatory pathways and suggested either that anti-TLR3 antibody is transported across the plasma membrane to endosomal TLR3, or a role for extracellular plasma membrane-associated TLR3 ¹⁵⁶. TLR3 has been observed by Kleinman et al., to bind small interfering RNA (siRNA) as well. In a murine model, 21-nucleotide siRNA caused phosphorylation of cell surface TLR3 on lymphatic endothelial cells as well as blood endothelial cells and induces apoptosis. Thus, TLR3 activation may jointly suppress blood and lymphatic neovascularization ¹⁵⁷. Further study of murine TLR3 stimulation

with siRNA induced retinal degeneration, and TLR3 is expressed on the cell surface of retinal pigment epithelial cells ¹⁵⁸.

I.9 ANALYSIS OF SERUM AND SALIVA

Blood has long been used in the diagnosis and monitoring of pathogenesis, whether the result of an exogenous infection or autogenous dysregulation. The formed elements of blood, erythrocytes, leukocytes, thrombocytes, as well as plasma and clotting factors, exist in ranges considered normal. When collected blood is allowed to clot, serum forms a distinct layer, separate from the formed elements and without clotting factors, or fibrinogens. Serum does contain antibodies, antigens, hormones, electrolytes, and if present, pathogenic species, drugs, and drug metabolites. Serologic analysis has been used in the investigation of periodontal disease as well, to further elucidate viral, immunologic, bacteriologic, and inflammatory mediatory profiles in the progression and treatment of the disease ^{128, 159, 160}.

Saliva is a diagnostic body fluid that may be non-invasively collected. It is used presently to diagnose disease, and in the investigation of malignant neoplasia, autoimmune disorders and hormonal analysis ¹⁶¹. Salivary testing offers great sensitivity and specificity in the diagnosis of HIV, HHV, CMV, EBV and hepatitis C virus ¹⁶², and characteristic alterations in salivary chloride and sodium concentrations may be diagnostic cystic fibrosis ¹⁶³. Saliva and GCF have been extensively studied in both SS and periodontal disease. Kinney et al., and Sexton et al., reported described a significant correlation of salivary and GCF biomarkers including MMP-8, MMP-9, osteoprotegerin,

C-reactive protein, IL-1beta, and MIP-1alpha, with clinical periodontal parameters. Both posited salivary analysis as a sensitive measure of periodontal disease progression ^{164, 165}.

CHAPTER II

COMPARISON OF SALIVARY AND SERUM LEVELS OF SOLUBLE TOLL- LIKE RECEPTOR 3 (TLR3) IN PATIENTS WITH SJÖGREN'S SYNDROME (SS) COMPARED TO AGE- AND SEX-MATCHED CONTROLS

II.1 SYNOPSIS

II.1.1 Background

This cross-sectional study examined potential differences in concentration of Toll-like receptor 3 (TLR3) in serum and citric acid-stimulated saliva from patients with Sjögren's Syndrome (SS) and healthy age-, sex-, and periodontal disease matched control patients.

II.1.2 Methods

Salivation was stimulated with 5.0 ml of 2% citric acid, and whole saliva samples were collected. Full-mouth clinical periodontal measurements were recorded from 21 SS patients and 22 unaffected control patients. Saliva and plasma TLR3 concentrations were determined by enzyme-linked immunoassays (ELISA). Data were tested statistically as appropriate to the data distribution.

II.1.3 Results

No difference in age, body mass index (BMI), salivary TLR3 concentrations, or clinical periodontal measurements were evident between groups. Salivary flow rates were significantly lower ($p < 0.001$) whereas plaque % were significantly higher ($p = 0.048$) in SS patients. Mean saliva and serum TLR3 levels were 0.183 ng/ml and

0.219 ng/ml, respectively, in SS patients. Mean saliva and serum TLR3 levels were 0.299 and 0.188 ng/ml, respectively, in healthy patients. There was no significant difference between serum or saliva in SS patients or healthy control patients.

II.1.4 Conclusion

The present findings do not support a hypothesis that a hyperinflammatory systemic state, as seen in treated SS patients, can independently raise serum or saliva TLR3 concentration in the absence of periodontal disease. This is the first report of TLR3 detection in human serum and saliva by ELISA.

II.2 INTRODUCTION

Sjögren's Syndrome (SS) is a chronic, systemic autoimmune disorder. The course of the disease destroys salivary and lacrimal glands, often causing xerostomia and xerophthalmia. Pulmonary, hepatic, renal, and neurologic extraglandular involvement may result as well. B-lymphocyte hyper-reactivity, autoantibody production and T-cell lymphocytic infiltration to exocrine glands, and commonly, other organs, are characteristic of SS pathosis, though the precise etiology, or etiologies, remains unknown¹.

Though the cause of the cellular activation and chronic inflammatory response that targets epithelial cells in SS has not been determined, a viral pathogenesis has long been suspected. Multiple studies have observed a correlation of viruses and SS. Elevated Epstein-Barr virus (EBV) levels in the blood, saliva, and salivary gland tissue of SS patients as compared to non-diseased controls are widely reported ^{104,105,106,107}. Human cytomegalovirus (HCMV), hepatitis C virus, coxsackie virus, and human T

lymphotropic virus type I, are among many viruses that have been investigated as causative and contributory factors as well¹⁶⁶⁻¹⁷¹. Viral ligands activate Toll-like receptors (TLRs) 3, 7, 8, and 9 resulting in exocrine gland alteration. Once activated, TLR pathways culminate in type 1 interferon (IFN-1) activation and chemokine production characteristic of the salivary gland immune response in SS¹⁷².

When bound to double-stranded RNA (dsRNA), TLR3 initiates an innate immune response¹³⁹, and may facilitate an adaptive immune response as well. dsRNA is a structure common to many viruses, and may be expressed during the replication of non-dsRNA viruses. Mast cells, macrophages, natural killer cells, B, Th1, Th2 and myeloid dendritic cells (DCs) all express TLR3.¹⁷³ Initially thought to be expressed exclusively intracellularly, it is likely expressed extracellularly as well^{140, 141, 142}. Ligation induces IFN genes and production of numerous inflammatory cytokines and chemokines, including IFN- α , IFN- β , TNF- α , IL-6, IL-12, IP-10, and RANTES^{174,148,148}.

Like SS, a viral pathogenesis for periodontal disease has been investigated. EBV, HCMV, and herpes simplex virus (HSV), as well as other common viruses have been implicated in the pathosis of periodontal disease. Virus-positive samples from subgingival plaque samples, gingival crevicular fluid, tissue biopsies, as well as serum and saliva samples have been collected and correlated with periodontal disease severity and frequency. EBV presence in periodontal lesions was positively correlated to *P. gingivalis* frequency¹³³. Periodontal lesions may be the primary origin of salivary HCMV, as well as a significant source of salivary EBV¹³². Viral pathogens have been detected in diseased periodontal tissue as well. EBV DNA has been observed in the

interproximal papilla¹²⁶, and junctional and sulcular epithelial cells of diseased individuals¹²⁷.

Though they may share a viral etiology, the relationship, if any exists, between SS and periodontal disease is unclear. Antoniazzi et al., and Najera et al., observed periodontal parameters such as probing depth, gingival and plaque index, clinical attachment loss, and bleeding on probing were significantly worse among SS patients as compared to SS-free patients^{81,82}. Conversely, Kuru et al., reported that the occurrence, severity and extent of periodontal disease as quantified by clinical parameters were not significantly different between pSS, sSS and SS-free patients⁸⁴. Schiødt et al., as well as others, concluded that pSS is not associated with increased risk of periodontal disease^{85-88,92}.

Blood has long been used in the diagnosis and monitoring of pathosis. When collected blood is allowed to clot, serum forms a distinct layer, containing antibodies, antigens, hormones, electrolytes, and if present, pathogenic species, drugs, and drug metabolites. Saliva contains many of the same elements as serum, and can also be used to diagnose and monitor pathosis¹⁶¹. Unlike serum, saliva can be non-invasively collected.

There are no studies to date that have investigated the presence and quantity of TLR3 in the saliva and serum of periodontally healthy patients with SS. Our hypothesis is that saliva and serum levels of TLR3 are increased in SS patients due to up-regulation by stimuli generated from receptor crosstalk involved in the signaling cascades of induction and progression of SS.

II.3 MATERIALS AND METHODS

II.3.1 Patient Selection

Forty-three subjects were included in the study. The study protocol was approved by the Texas A&M Health Science Center Institutional Review Board, and conducted in full compliance with ethical principles, including the 2000 revision Helsinki Declaration of 1975. Twenty-one non-smoker, SS-positive patients and twenty-two, non-smoking, age and sex-matched control patients were recruited. SS patients were recruited from October 2012 to August 2013 from the Stomatology and Salivary Dysfunction Clinic at TAMU Baylor College of Dentistry (TAMUBCD). Control patients were recruited from the faculty and staff of Texas A&M University-Baylor College of Dentistry. A positive SS diagnosis was made by satisfying the American-European Consensus Classification Criteria ³⁸. Prior to salivary and venous blood sampling, and clinical periodontal examination, the study protocol was explained, and written informed consent, medical and dental histories were obtained. No less than 16 teeth including at least 2 molars, were present in each patient. Exclusion criteria were diabetes mellitus, hepatitis, malignancies, pregnancy and/or lactation, less than 18 years old, or periodontal therapy within the previous 6 months. The clinical case definitions proposed by the Centers for Disease Control working group for use in population based surveillance of periodontitis were used for periodontal disease assessment ¹⁷⁵. Patients with neither more than 1 interproximal site with clinical attachment loss of at least 3 mm, nor more than 1 interproximal site with probing depth of at least 4 mm, were considered periodontitis-free. Following SS patient data collection, age and sex-matched

control patients were recruited and appropriately excluded according to the aforementioned criteria. This effort was made to exclude or minimize any effect periodontal disease may have on serum and salivary levels of TLR3.

II.3.2 Saliva Collection

Stimulated saliva was collected as described by Navazesh et al.¹⁷⁶. Patients were instructed to brush, floss, rinse with water, and fast for two hours prior to saliva collection. Immediately before collection, patients rinsed with 10 ml of sterile deionized water for 1 minute and expectorated. A five second swish of 5 ml of 2% citric acid followed to stimulate salivary flow. After 1 minute, the patient again expectorated to void any accumulated secretions. Expectorated whole saliva was then collected on ice in sterile pre-weighed 50.0 ml polypropylene tubes until 5.0 ml was collected or 20 minutes had elapsed. The collection period was timed to allow for salivary flow rate calculation, and the determination of the captured sample weight. Collected saliva was immediately centrifuged (800g) for 10 minutes at 4°C, then aliquoted into 500 µl portions. These portions were then frozen and stored at -80°C until all study samples were collected. All portions were thawed immediately prior to assay.

II.3.3 Serum Collection

10 ml of venous blood was collected by standard venipuncture with vacutainers^A from the antecubital fossa or superficial veins of the hand. Immediately after collection, the vacutainer was inverted 5 times and then left undisturbed for 1 hour per manufacturer's recommendation. 12,000 rpm centrifugation for 10 minutes separated

^A BD Biosciences, North America

serum from blood. The serum was then frozen and stored at -80°C until all study samples were collected. All samples were thawed immediately prior to assay.

II.3.4 Periodontal Examination

Following saliva and serum collection, clinical periodontal parameters including probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) were recorded with an UNC15 probe^B. BOP was positive if it occurred within 30 seconds following probing. CAL was calculated as the distance between the base of a probable pocket and the cement-enamel junction. A Nabors probe and bi-digital manipulation determined the degree of furcation involvement (Glickman classification) and mobility (Miller classification) if present, respectively. A disclosing agent^C was applied per manufacturer's recommendation, and plaque was recorded dichotomously as present or absent at six sites per tooth, including mesial-, mid-, and distal- labial and lingual surfaces. Two examiners (HP, JB), calibrated prior to and three months after following the study initiation, recorded all measurements. Demographic and periodontal measurements were reported for all patients in the study (n=21 SS, n=22 control).

II.3.5 Measurement of TLR3 in Serum and Saliva

Two samples, one test and one control, used for calibration purposes were not included in ELISA analysis. The remaining 20 test samples and 20 randomly picked (coin flip) control samples were selected for analysis with TLR3 enzyme-linked

^B University of North Carolina Periodontal Probe, Hu-Friedy, Chicago IL.

^C Young 2-Tone™, Earth City, MO

immunosorbent assay (ELISA) kits^D following the manufacturer's protocol. The average optical density (OD) of each undiluted saliva and serum sample was compared to the assay standard curve concentrations to determine TLR3 concentration. A linear equation derived from the standard curve and measured OD values was used to derive TLR3 concentration in samples with OD beyond the maximum detectable range. The lower and upper detection thresholds for TLR3 assay were 0.016 ng/ml and 10.0 ng/ml, respectively.

II.3.6 Statistical Analysis

Shapiro-Wilk normality tests were performed on all numerical variables with the null hypothesis that the variable in question was normal versus the alternative that the variable was not normal. For variables that were normal, a t-test was performed between the control group and the SS group. For variables that were not normal, Mann-Whitney was used, and to settle disputes caused by ties in Mann-Whitney, jittering was used and repeated (Monte-Carlo) with 10,000 permutations. The p-value was averaged over the 10,000 permutations, creating a simulated p-value, and reported. Correlation coefficients were found using Pearson's correlation coefficient between variables, and correlation coefficients greater than 0.6 were considered to be noteworthy. Finally, for the binary data if the patient visited the dentist in the past 6 months (1 = Yes and 0 = No), the Fisher's Exact Test was used because this data was structured as a 2x2 contingency table.

^D Boster Immunoleader, Pleasanton, California, USA.

The number of patients taking an individual medication was counted and summed according to the therapeutic category the medication belonged to (e.g. antihypertensives, psychotics, biologics, etc.). A percent difference was calculated as:

$$\frac{(\text{number of SS patients taking drug} - \text{number control patients taking drug})}{(\text{number of SS patients taking drug})}$$

The distribution of the number of drugs taken by patients was tested for normality using the Shapiro-Wilk statistic, which indicated that the distribution was in fact not normal. Therefore, the total number of drugs used by the SS group and control group was analyzed using a Mann-Whitney test to see if there was a significant difference.

Serum and saliva were analyzed for TLR3 using a Mann-Whitney test for analysis. Twenty patients were used for each group because of the cost of measuring TLR3. Two patients from the control group were subsequently randomly discarded and one patient from the test group was also discarded. All tests were performed at alpha = 0.05. All statistical tests were completed with SPSS (IBM).

II.4 RESULTS

II.4.1 Clinical Results

There were no significant differences between the examiners for measured clinical variables (Table 1, Figure 1). Table 2 reports the mean and median values obtained for all clinical variables. SS and control groups did not differ significantly ($p>0.05$) in age, BMI, PD, CAL, BOP, mobility, PD \geq 4 mm, furcation involvement, or dental care, with both groups reporting at least semiannual dental care. Conversely,

salivary mass and salivary flow were significantly lower ($p < 0.001$), and plaque percentage significantly higher ($p < 0.049$) in SS group as compared to controls.

As compared to the control group, a greater number of SS group patients took drugs in 11 of 14 drug categories (Table 3). A greater number of control group patients took drugs belonging to antihypertensive, antiplatelet and allergy medications. However, the amount of medication to treat allergies, hypertension, and hyperlipidemia were not significantly different between groups. SS patients took significantly more medications to treat hyperlipidemia, psychological conditions, xerostomia, pain, gastro-esophageal reflux disease, insomnia, thyroid dysregulation, hormone replacement therapy, and anti-malarial agents. On average, patients belonging to SS group took 6.61 drugs as compared to 2.36 drugs taken by control patients.

Correlations were calculated. Correlations with $r > .6$ were determined to be relevant. Relevant correlations include Flow with Saliva Mass ($r = .863$), Flow with Collection Time ($r = -.759$), Average PD with Average CAL ($r = .604$), PD > 5 mm with Collection Time ($r = .655$), and number of Grade 1 Furcation with Grade 2 Furcation involvement ($r = .617$). The variables that showed significance with the appropriate hypothesis testing ($\alpha = .05$) were Salivary Mass ($p < .001$), Salivary Flow ($p < .001$), and plaque index ($p = .049$). Age showed moderate significance between the two groups ($p = .085$). The 3:18 ratio of men-women in the SS group of the study reflected the population proportion of 1:9 accurately, with no significant difference between the study and the true population proportion. A z-proportion test result of $p = .735$ confirmed this finding.

II.4.2 Saliva and Serum TLR3 Concentration

There was no statistically significant difference in saliva or serum TLR3 levels between patient groups ($p = 0.465$ and 0.556 , respectively) (Table 4).

A post-hoc power analysis was performed using the observed ELISA results. The observed effect sizes for serum and saliva were 0.11 ng/ml and 0.16 ng/ml, respectively.

II. 5 DISCUSSION

With the exception of the percentage of plaque present, the clinical periodontal parameters reported in this study were not significantly different between SS and control groups. PD, CAL, BOP, mobility, $PD \geq 4$ mm, furcation involvement, and dental care, were all similar. The periodontal disease status of SS patients as compared to disease free controls was grossly equivalent. This finding is in agreement with the observations of multiple authors^{84-86, 88}. Kuru concluded that among pSS, sSS and SS-free patients, PI, GI, BOP, PD, CAL, and occurrence, severity and extent of periodontal lesions were not significantly different⁸⁴. Boutsis et al., found no difference in the clinical periodontal parameters between pSS/sSS patients and healthy controls⁸⁸. With few exceptions, Jorkjend reported no statistically significant difference between the periodontal status of SS and healthy controls, concluding that SS patients do not have an increased risk for developing periodontitis⁹⁰.

However, not all studies have produced similar observations. Najera et al., reported significantly higher PI, increased alveolar bone loss, CAL and cement-enamel junction to-bone crest loss among SS patients. An odds ratio analysis of this data placed SS patients at a 2.2 times higher risk of periodontal disease than SS-free individuals⁸².

In accord with Najera, Antoniazzi et al., posited that SS patients had significantly higher PI, GI, PD, CAL, and BOP ⁸¹.

The single significantly different clinical parameter between SS and control groups was plaque, with 25.6% and 19.5% of surfaces plaque-positive, respectively. Whether a 6% difference is clinically significant is subject to debate. Furthermore, because the method of plaque recording made no quantification of the amount of plaque, but only presence or absence, the biologic burden presented to the periodontium may not be accurately reflected by this metric.

The mass of collected saliva and salivary flow were significantly different between groups as well, with SS patients having significantly lower mass and flow. This finding of relative hyposalivation, and the oft-accompanying symptom xerostomia is well-documented ^{6, 62}. Decreased salivary output might explain the observed significant difference in plaque.

By the very nature of the study design, which selected SS patients who were properly diagnosed and treated, poorly managed SS patients were likely excluded. Because it is unethical to demand patients cease successful therapy, or discontinue use of any medication for the purpose of an investigation, it is possible that the continued pharmacologic management of SS by patients may alter TLR3 expression. SS patients took significantly more medications to treat hyperlipidemia, psychological conditions, xerostomia, pain, gastro-esophageal reflux disease, insomnia, and thyroid dysregulation, as well as medications for hormone replacement therapy and anti-malarial agents.

Because SS disproportionately afflicts peri- and post-menopausal females, many women suffering from SS may receive hormone replacement therapy (HRT). One-half of SS patients in this study reported HRT. The expression of TLR3 mRNA is known to fluctuate during the menstrual cycle, and may be correlated to hormonal fluctuation. Analysis of endometrial biopsies obtained throughout the human menstrual cycle show significant variance in TLR3 mRNA expression. TLR3 mRNA expression is significantly higher in the early proliferative phase than the late proliferative phase, and is significantly higher in the late secretory phase than the late proliferative and early secretory phase ¹⁷⁷. Indeed, two case reports of SS following initiation of estrogen HRT have been reported ¹⁷⁸. Furthermore, Jones et al., investigated progesterone modulation of murine bone marrow-derived dendritic cell cytokine production (IL-6 and IL-12) and costimulatory molecule expression (CD40, CD80, and CD86) induced by TLR3. Progesterone was found to downregulate TLR3 -induced IL-6 production via the glucocorticoid receptor, and downregulated IL-12p40 production via either the glucocorticoid receptor or progesterone receptor. Progesterone also significantly inhibited TLR3-CD40 expression in bone marrow-derived DCs ¹⁷⁹. Though not definitive, much evidence suggests hormonal levels impact TLR3 expression.

Nine of 20 SS patients reported the use of the anti-malarial drug hydroxychloroquine. Anti-malarial agents are known to alter immune regulation and response, and are used therapeutically in multiple autoimmune disorders. Chloroquine and quinacrine efficiently inhibited production of inflammatory cytokines in pDC and PBMCs incubated with the immune complexes of sera from systemic lupus

erythematous patients. Chloroquine and quinacrine had inhibitory effects on TLR3 signaling when these receptors were stimulated with nucleic acids. Poly(I:C) was unable to trigger activation of TLR3 due to changes in conformation of nucleic acids following interaction with chloroquine with nucleic acids. Although conducted in vitro, this provides evidence of TLR3 alteration when anti-malarial drugs are present ¹⁸⁰.

To date, the authors are unaware of any study that analyzed human serum or saliva for TLR3 by ELISA. Although no significant difference in the serum or saliva TLR3 levels between SS and healthy controls was observed, it was previously unknown whether TLR3 could be detected. Therefore, the data generated may serve to establish baseline or normative values. Although TLR3 expression should correlate with the level of viral dsRNA, and thus viral infection, it is an indirect indicator.

A post-hoc power analysis was performed using the observed ELISA results. Because this study was novel, an a priori power analysis could not be conducted, and a power analysis for a study of TLR4 conducted in parallel was used. The observed effect sizes for serum and saliva TLR3 were 0.11 ng/ml and 0.16 ng/ml, respectively. Hypothetically, the effect sizes for serum and saliva would be 0.232 ng/ml and 0.314, respectively, when achieving a power of 0.8. This would necessitate 294 and 160 age- and sex- matched study participants for serum and saliva, respectively. As such, the findings of this study should not be interpreted as evidence of an absence of viral pathosis in SS. Rather, additional investigation with a greater sample size is necessary.

CHAPTER III

CONCLUSION

This is the first study to detect, and quantify, TLR3 by ELISA in human serum and saliva samples. The values recorded in this study may be applied to establishing normative TLR3 values. The present study observed that SS patients had comparable periodontal status when compared to age- and sex-matched controls as quantified by clinical parameters. SS patients demonstrated significantly greater plaque percentage and significantly lower salivary flow rate. Serum and saliva levels of TLR3 did not differ significantly between SS and control groups.

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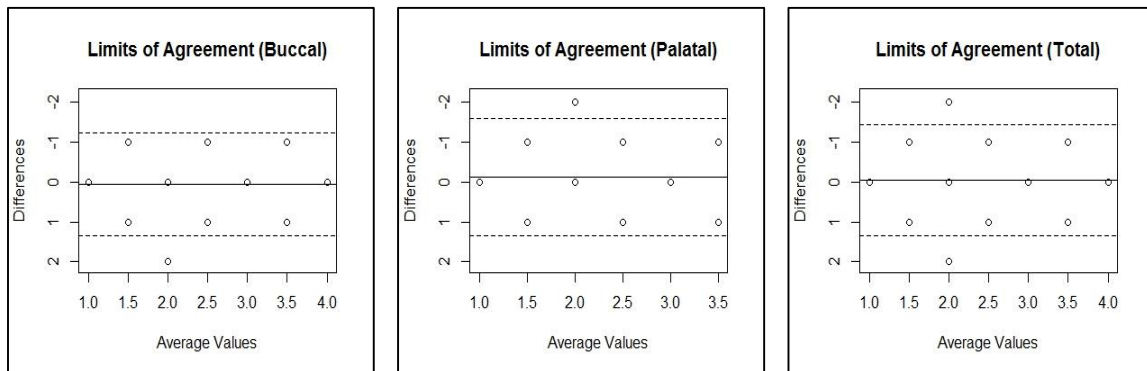
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APPENDIX A

FIGURES

FIGURE 1: LIMITS OF EXAMINER AGREEMENT



Bland-Altman Plots. The dotted lines in each plot is the 95% confidence interval margins around the bias. The buccal and palatal plots show one point outside the bands. The total plot shows the combination of the palatal and buccal plots.

APPENDIX B

TABLES

TABLE 1: CALIBRATION STATISTICS

	Buccal	Palatal	Total
Percent Agreement	65.7	54.3	60.0
T-test p-value	.459	.122	.496
ICC	.628	.491	.558
T-test p-value (Bias)	.229 (0.047)	.519 (-0.114)	.750 (-0.033)

Both raters were similar. The total percent agreement was above 60%, and the total t-test for the raw difference between both raters was not significant (p-value = .496). Additionally, the t-test for bias was insignificant (p-value = .75).

TABLE 2: PATIENT DEMOGRAPHICS, SALIVA MEASUREMENTS, AND PERIODONTAL MEASUREMENTS

	SS Patients n = 21 Median (IQR)*	Control Patients n = 22 Median (IQR)*	p-value
Age (years)*	59.3 (12.07)	52.6 (12.99)	0.085
Saliva Mass (g)*	3.31 (1.48)	4.99 (1.37)	<0.001
Salivary Flow (g/min)	0.30 (0.19)	0.89 (0.57)	<0.001
Body Mass Index*	28.6 (7.24)	29.9 (7.53)	0.549
Average Probing Depth (mm)*	2.27 (0.42)	2.33 (0.25)	0.552
Average Clinical Attachment Loss (CAL) (mm)*	2.58 (.58)	2.50 (0.41)	0.569
Bleeding on Probing	1.00 (8.00)	0.50 (2.00)	0.225
Plaque %	25.6 (35.90)	19.5 (10.00)	0.049
Class 1 Mobility (# teeth)	0.00 (0.00)	0.00 (0.00)	0.547
Class 2 and 3 Mobility (# teeth)	N/A	N/A	N/A
Probing depth ≥4mm (# teeth)	0 (4.00)	0 (1.00)	0.568
Probing depth ≥5mm (# teeth)	0 (0.00)	0 (0.00)	0.423
Grade 1 Furcations (# teeth)	1.00 (2.00)	0.00 (1.75)	0.397
Grade 2 Furcations (# teeth)	0.00 (0.00)	0.00 (0.00)	0.408
Grade 3 Furcations (# teeth)	N/A	N/A	N/A
Dental visits in last >2 times per year (% of group who did)**	67	54	0.132

The single star (*) indicates a variable that was determined normal by Shapiro-Wilk statistic. For each normal variable the mean and standard deviations were reported. Additionally, a t-test was performed, and its associated p-value was reported. For those not marked with a single star (*) or a double star (**), the median and interquartile range (IQR) was reported, as well as the p-value from the Mann-Whitney test. Finally, the final row (designated as “**”), the percent of patients who did visit the dentist within 6 months and the p-value from Fisher’s exact test was reported.

TABLE 3: MEDICATION INFORMATION FOR CONTROL AND SS GROUPS

Medication	# Medications in SS patients	# Medications in Control Patients	Percent Difference (%)
ANTIHYPERTENSIVES	8	15	-87.5
• Beta-blockers	2	4	
• Diuretics	1	6	
• Ca++ blockers	4	2	
• Angiotensin 2 inhibitors	1	3	
ANTI-PLATELET	3	5	-66.7
• Salicylic Acid	3	3	
• Clopidrigel	0	2	
ANTI-HYPERLIPIDEMICS	6	4	33.3
GASTROESOPHAGEAL REFLUX DISEASE	11	5	54.5
• Proton Pump Inhibitors	9	5	
• Antacids	2	0	
PYSCHOLOGIC	10	5	50.0
• Selective serotonin reuptake	5	2	
• Norepinephrine reuptake	1	1	
• Lithium	2	1	
• Tricyclic anti-depressants	1	1	
• Atypical anti-psychotic	1	0	
ANALGESICS	28	11	60.7
• Nsaids	8	4	
• Barbituates	1	0	
• Opiods	5	1	
• Systemic Steroids	1	0	
• Muscle Relaxers	6	3	
• Anti-migraine	1	1	
• Gabapentin	3	1	
• Cox-2 Inhibitors	0	1	
• Pregabalin	4	0	

TABLE 3: CONTINUED

Medication	# Medications in SS patients	# Medications in Control Patients	Percent Difference (%)
ALLERGY	5	7	
• Leukotriene receptor antagonist	1	1	
• Histamine receptor antagonist	0	2	
• Nasal corticosteroid	3	2	
• Inhaled corticosteroid	1	1	
• Ophthalmic antihistamine	0	1	-40.0
HORMONE REPLACEMENT THERAPY	10	1	
• Estrogen receptor agonist	9	1	90.0
• Progesterone receptor agonist	1	0	
HYPOTHYROID	7	2	71.4
SIALOGOGUES	18	0	100.0
INSOMNIA	10	0	
• Benzodiazepines	6	0	100.0
• Zolpidem	4	0	
ANTI-MALARIALS	9	0	100.0
MISCELLANEOUS	6	2	
• Ophthalmic drops for dry eye	4	1	
• Bladder-selective Alpha-1 antagonists	0	1	66.7
• Bisphosphonates	2	0	
BIOLOGICS	1	0	100.0

TABLE 3: CONTINUED

Total # medications	117	57	51.3
Average # medications/pt	6.61	2.36	64.3

This table shows the aggregate number patients taking drugs and types of drugs. The bolded numbers along with the bolded and capitalized category shows the total number of patients taking the drugs listed per the specific test group status.

TABLE 4: SALIVARY AND SERUM TLR3 LEVELS FOR SS AND CONTROL GROUPS AND ASSOCIATED P-VALUES

	SS Patients	Control Patients	
	n = 20	n = 20	p-value
	Median (IQR)	Median (IQR)	
Salivary TLR3 (ng/mL)	0.183 (.440)	0.299 (.758)	0.465
Serum TLR3 (mg/mL)	0.219 (.426)	0.188 (.387)	0.556